Difference in pathway of *Escherichia coli* outer membrane permeation between penicillins and cephalosporins

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The differences in the outer membrane permeation between two major subgroups of β-lactam antibiotics were studied. The permeation of cephalosporins was closely related to porin channels in the outer membrane. In contrast, the outer membrane permeation of penicillins did not decrease in porin-deficient mutants and, in Rd-type mutants, their permeability became proportional to the hydrophobicity of the molecules. The activation enthalpy of the penicillin permeation was significantly larger than that of cephalosporins. These observations indicate that penicillins can use the hydrophobic region for the major route of outer membrane passage whereas the cephalosporin permeation is restricted to the pathway via the porin pore.

Penicillin *Cephalosporin* *β-Lactam antibiotic* *Membrane permeation*

1. INTRODUCTION

β-Lactam antibiotics must permeate the outer membrane of gram-negative bacteria to reach their target [1,2]. It has been claimed by many workers that β-lactam antibiotics use porin channels as a pathway for their outer membrane passage [3–5]. This concept was also supported by the fact that mutants lacking the Omp F and Omp C proteins in *Escherichia coli* cause a significant decrease in bacterial susceptibility to cephalosporins [3,6]. On the other hand, it is known that the alteration in bacterial susceptibility to ampicillin in the porin-deficient mutants is evidently smaller than the case of cephalosporins [3,6,7], suggesting the presence of an additional pathway other than porin channels for penicillin permeation [7]. An interesting fact is that such a difference in the porin-deficient mutants between cephalosporins and penicillins does not seem to be based on the difference in their hydrophobicity or molecular size [3,6,7]. In [8,9], we demonstrated that penicillins such as ampicillin and benzylpenicillin could permeate the phospholipid bilayer membrane very much faster than cephalosporins possessing similar hydrophobicity.

This study was carried out to confirm that penicillins can use pathways other than the porin channels for the major route of outer membrane passage in *E. coli*.

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

E. coli YA21 (a derivative of *E. coli* K12; F^-,met,leu,λ^-) and YA21-6 ($T4^r$ mutant of YA21 with heptose-deficient LPS [10,11]) were generous gifts from S. Mizushima. *E. coli* YA21C1 is a spontaneous mutant lacking Omp C and Omp F, and isolated from YA21 on the basis of cefoxitin resistance. To assay outer membrane permeation of β-lactam antibiotics by the method utilizing periplasmic β-lactamase [1], we infected the 3 *E. coli* strains with an R plasmid, RGN823, which mediates type Ib β-lactamase (TEM-2 β-lactamase) production [7].

2.2. *β-Lactam antibiotics*

β-Lactam antibiotics were kindly provided by the following pharmaceutical companies: benzylpenicillin and ampicillin, Meiji Seika Co., Tokyo; ceftezole and cefazolin, Fujisawa Pharmaceutical Co., Osaka; cephalexin and piperacil-

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lin, Toyama Chemical Co., Tokyo; cephalothin, Torii Pharmaceutical Co., Tokyo; cefadroxyl, Bristol and Banyu Pharmaceutical Co., Tokyo; apalcillin, Sumitomo Chemical Co., Osaka.

2.3. *Determination of outer membrane permeability parameter of β-lactam antibiotics*

Exponentially growing cultures of *E. coli* in 200 ml of penassay broth (Difco Co.) were harvested by centrifugation for 15 min at 5000 × *g* at 20°C. Cells were washed once with 50 ml of phosphate-buffered saline (pH 7.0) containing 1 mM magnesium sulfate and resuspended in 20 ml of the same buffer. A portion of this suspension was sonicated for 2 min at 0°C with a Branson cell disruptor model 200 in order to liberate the periplasmic β-lactamase. This suspension was used to measure the velocity of β-lactam hydrolysis by disrupted cell suspensions ($v_{disrupt}$). The rest of the cell suspension was directly used for the assay of the velocity of hydrolysis by intact cell suspensions (v_{intact}). Just after the assay of v_{intact}, the intact cell suspension was quickly centrifuged for 1 min by an Eppendorf centrifuge model 5412 and β-lactamase activity of the supernatant was measured (v_{sup}). Hydrolysis of β-lactam antibiotics was measured by a modified microiodometric method as in [1].

The permeability parameter C (= PA) was calculated using Fick's law of diffusion by the method described in [14], which was modified as follows:

$$V_{max} = (1 + K_m/S_o) \cdot v_{disrupt} \quad \text{(i)}$$

$$S_i = K_m \cdot v_{intact}/(V_{max} - v_{intact}) \quad \text{(ii)}$$

$$C = V_{max} \cdot S_i/(K_m + S_i)/(S_o - S_i) \quad \text{(iii)}$$

where S_o and S_i are concentrations of β-lactam antibiotics in the medium and in the periplasmic space of the cells, respectively, at the steady state. The permeability parameter was normalized by the dry weight of cells in the assay mixture.

2.4. *SDS-polyacrylamide gel electrophoresis of outer membrane proteins*

Crude membranes were prepared by sonic disruption of the mid-log-phase cells as in [7] and then incubated in 10 mM phosphate buffer (pH 7.0) containing 2% sarkosyl NL-97. The pellet, after centrifugation at 100000 × *g* for 40 min at 10°C, was washed twice with 1% sarkosyl solution and used as the outer membrane preparation. SDS-polyacrylamide slab gel electrophoresis of the outer membrane in the presence of 8 M urea was performed by a modification of the method of Mizushima and Yamada [12]. Thin (0.8 mm) slab gels were composed of 8% acrylamide, 0.13% *N,N*-methylenebisacrylamide, 0.5% SDS, 8 M urea and 0.1 M sodium phosphate (pH 7.2). 50 μl of outer membrane suspension (250 μg protein) was mixed with 200 μl of 1.25% SDS-1.25% β-mercaptoethanol solution and heated in boiling water for 5 min and then 185 mg of urea and 25 μl of 1% BPB was added to the mixture. Ten μl of the mixture was loaded on the slab gel and the electrophoresis was carried out as in [7].

3. RESULTS AND DISCUSSION

3.1. *Outer membrane components of the strains used in this experiment*

E. coli YA21 is a wild-type strain with regard to the outer membrane [13], while *E. coli* YA21-6 is a mutant completely lacking heptose in its lipopolysaccharide chain (Rd mutant) (fig.1) [11,13]. As is obvious from the SDS-PAGE patterns in fig.2, *E. coli* YA21 had both porin proteins Omp F and Omp C, and the amount of Omp F protein was somewhat larger than that of Omp C protein. Only a trace amount of porins could be detected in the SDS-PAGE patterns of outer membrane proteins from cefoxitin resistant strain YA21C1. Rd mutant YA21-6 also had both Omp F and Omp C porins, however, in contrast to *E.*

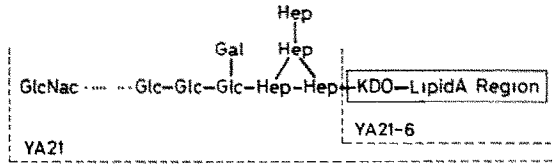

Fig.1. The structure of the oligosaccharide region of lipopolysaccharide of *E. coli* YA21 (wild type) and YA21-6 (Rd type). Data were taken from Yu and Mizushima [13]. GlcNAc, *N*-acetylglucosamine; Glc, glucose; Gal, galactose; Hep, heptose; KDO-Lipid A Region, the region containing 2-keto-3-deoxyoctanoate and lipid A.

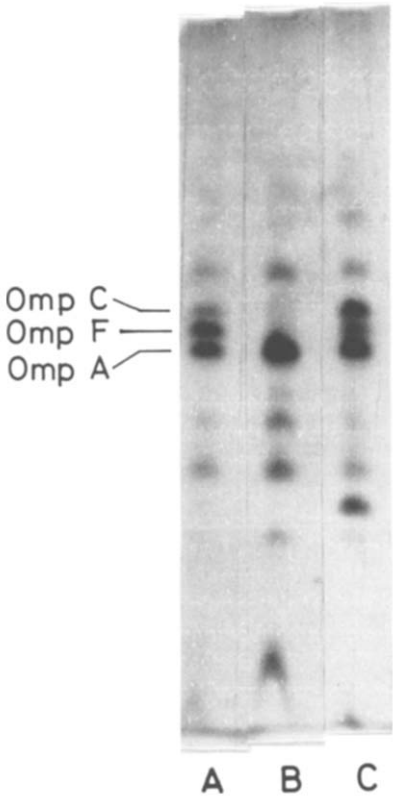

Fig.2. Gel electrophoretic profiles of outer membrane proteins of *E. coli* YA21, YA21C1 and YA21-6. Gel electrophoresis was performed as described in section 2. Lane A, YA21; lane B, YA21C1; lane C, YA21-6.

coli YA21, the amount of Omp F porin was smaller than that of Omp C porin.

3.2. *Dependency of outer membrane permeation of penicillins and cephalosporins on the hydrophilicity of the molecules*

Fig.3 shows the relationship between outer membrane permeability of β-lactam antibiotics and hydrophilicity of the drugs, which was compared between *E. coli* YA21 and its porin-deficient mutant YA21C1. In the wild strain YA21, the permeability was directly proportional to their hydrophilicity, though the dependency for penicillin permeation on hydrophilicity was smaller than the case of cephalosporins. The permeability of cephalosporins in the porin-deficient mutant YA21C1 was lower by a factor of one order or more when compared with the wild strain. Such a reduced permeation of cephalosporins in the mutant was even proportional to the hydrophilicity of the molecules. Thus the permeation of cephalosporins in the porin-deficient mutant was probably performed by the trace amount of porins remaining.

In contrast, penicillins showed higher permeability in the porin-deficient mutant than in the wild-type strain (fig.3). The increased permeability in the mutant strain was also propor-

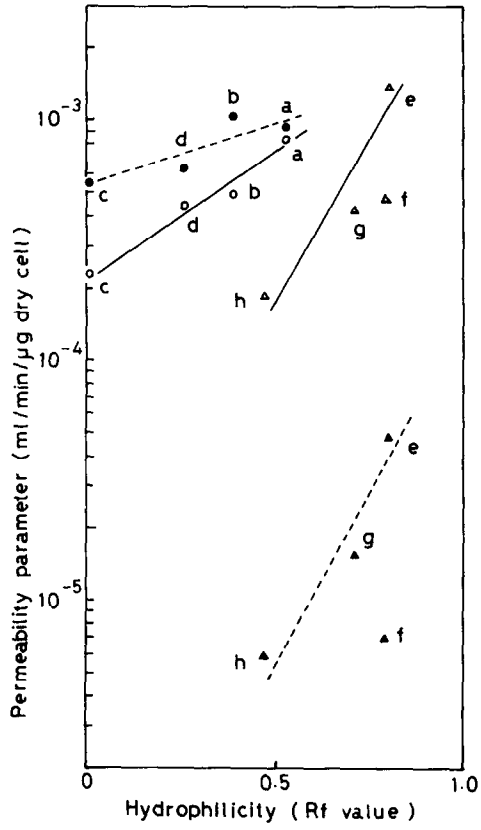

Fig.3. Outer membrane permeability of β-lactam antibiotics in *E. coli* YA21 and YA21C1 (porin-deficient). Outer membrane permeability was measured as described in section 2. R_f was determined by reversed-phase thin layer chromatography as in [9]. Triangles represent cephalosporins and circles represent penicillins. Open symbols with solid lines denote the permeability to YA21 and closed symbols with broken lines represent that to YA21C1. a, ampicillin; b, benzylpenicillin; c, apalcillin; d, piperacillin; e, ceftezole; f, cefadroxyl; g, cefazolin; h, cephalothin.

tional to the hydrophilicity of penicillins. Such a phenomenon could not be explained by the known concept that penicillins only use porin channels as the route for outer membrane permeation. It has been reported that the mutational deletion of major outer membrane proteins causes disorder of the outer membrane structure resulting in the appearance of a phospholipid layer on the outer leaflet of the outer membrane [14]. The increase in permeability in the mutant strain became progressively greater as hydrophobicity of penicillin was raised, and this phenomenon might be due to the phospholipid layer permeation. However, the absolute permeability of penicillins was proportional to their hydrophilicity even in a porin-deficient mutant. Such a complex phenomenon may be attributed to the barrier function of the polysaccharide chain layer on the surface of the outer membrane. In [7], we demonstrated an interesting fact by using an artificial membrane vesicle composed of bacterial phospholipids and lipopolysaccharides from *E. coli* that the polysaccharide chain layer on the vesicle acts as a barrier against the permeation of hydrophobic solutes into the vesicle.

To elucidate the role of polysaccharide chains in the outer membrane permeation of β-lactams, we measure the permeability of β-lactams in Rd mutant YA21-6 which lacks most of the polysaccharide chains [13]. As shown in fig.4, the outer membrane permeability of penicillins in the mutant was clearly proportional to hydrophobicity of the drugs. On the other hand, cephalosporins showed permeability which was still proportional to their hydrophilicity even in the Rd mutant. It should be noted that the total amount of porin proteins in the Rd mutant was about the same as that of the wild strain though the amount of Omp F porin was somewhat lower than the wild type. The results obtained with the Rd mutant strongly suggest that the permeation via the hydrophobic region was predominant in the outer membrane permeation of penicillins in *E. coli*. However, this concept is in disagreement with the common knowledge that the usual penicillins pass through the outer membrane via the porin pore.

There may be another criticism that the high permeability of penicillins in the outer membrane mutants is attributed to the unusual structure of the outer membrane and there is no reason for a similar phenomenon occurring in the normal outer membrane.

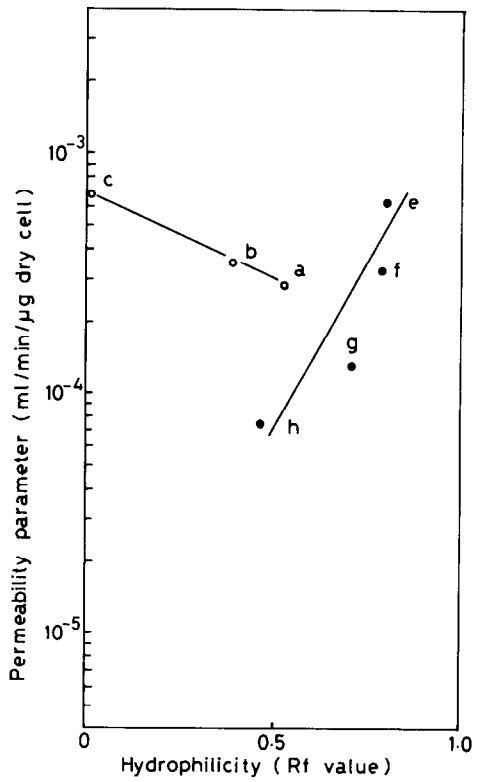

Fig.4. Outer membrane permeability of β-lactam antibiotics in *E. coli* YA21-6 (Rd mutant). Permeability and R_f were measured as in fig.3. Open and closed circles represent the permeability of penicillins and cephalosporins, respectively. Abbreviations are the same as in fig.3.

3.3. *Temperature dependency of outer membrane permeability of β-lactams*

To investigate the permeation route of β-lactams from a physicochemical point of view, the temperature dependency of the permeability was measured. If a solute permeates through the hydrophobic region of the outer membrane, the enthalpy of the permeation should be higher than that of the permeation through a water-filled porin pore, because dehydration of a solute is required for dissolving into a hydrophobic region [15]. The enthalpy necessary to pass through the porin pore could be roughly estimated to be equal to that of the diffusion in bulk water phase, which is around 1.3 kcal/mol [15]. On the other hand, the enthalpy

of the permeation through the phospholipid bilayer is more than 10 kcal/mol and increases in proportion to the hydrophilicity of the molecule [16].

As shown in fig.5, the outer membrane permeability of the hydrophobic penicillin, apalcillin showed the same temperature dependency in the wild strain as in the porin-deficient mutant. The enthalpy for the apalcillin permeation was 16–17 kcal/mol (table 1), which is about the same as that for the lipid bilayer permeation. On the other hand, the enthalpy for the outer membrane permeation of cefazolin was significantly smaller than that of apalcillin (table 1). If cefazolin utilizes the hydrophobic region as the major route of outer membrane permeation, the enthalpy for its permeation should be larger than that of apalcillin because cefazolin is more hydrophilic than apalcillin. However, the value found was of about the same order as that for the diffusion in bulk water phase. These results suggest that cefazolin and apalcillin permeate the outer membrane of wild strain via different pathways, i.e., water-filled porin pore and hydrophobic region composed of hydrocarbon chain, respectively.

The temperature dependency of ampicillin permeation in the wild strain was significantly greater than the case of cefazolin permeation (fig.6). The enthalpy for ampicillin permeation was estimated to be 24 kcal/mol which is also larger than that for apalcillin permeation. Such a higher enthalpy for ampicillin permeation may be reasonable when it permeates through hydrophobic regions, because ampicillin is more hydrophilic than apalcillin. These results suggest that the pathway through the hydrophobic region plays a great role in the outer membrane permeation of penicillins even in the normal outer membrane, whereas cephalosporins are restricted to the pathway via porin pore.

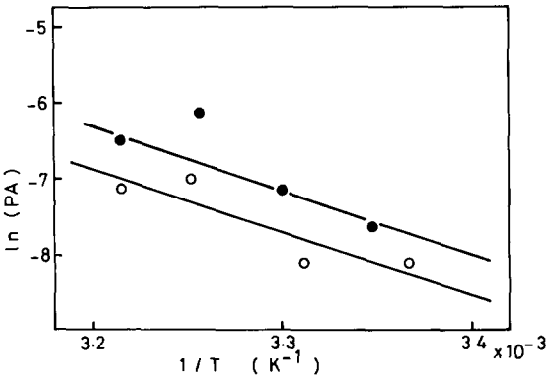

Fig.5. Temperature dependence of outer membrane permeability of apalcillin in *E. coli* YA21 and YA21C1. The permeability was measured as described in section 2. Open and closed circles represent the permeability to YA21 and YA21C1, respectively.

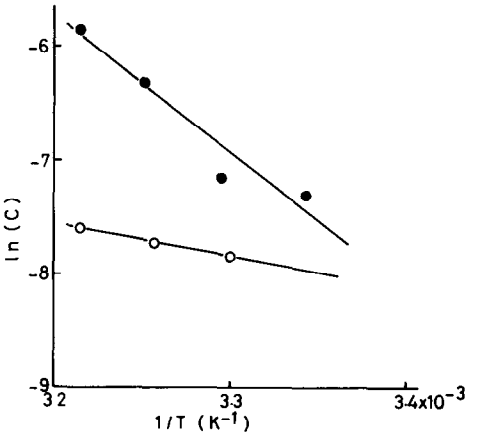

Fig.6. Temperature dependence of outer membrane permeability of ampicillin and cefazolin in *E. coli* YA21. The permeability was measured as in fig.6. Open and closed circles represent the permeability of cefazolin and ampicillin, respectively.

On the basis of the β-lactam resistance of porin-deficient mutants, Jaffe et al. [6] and Alphen et al. [3] assumed that ampicillin permeates the outer membrane mainly via the Omp F porin channel rather than the Omp C porin channel, whereas cephalosporins permeated via both Omp F and Omp C porin channels on the basis of the β-lactam resistance of porin-deficient mutants. However, it should be noted that the increase in ampicillin resistance of the porin-deficient mutants was significantly smaller than that in cephalosporin resistance [3,6]. We also found a similar phenomenon [7]. These observations indicated the presence of an additional pathway for outer membrane permeation of ampicillin. Here, we offered additional evidence that penicillins involving ampicillin can use the hydrophobic region as a major route for its outer membrane permeation in contrast to cephalosporin, the permeation route of which is restricted to porin channels.

On the other hand, there is the conflicting fact that, in reconstituted outer membrane vesicles, ampicillin can permeate through the porin channel depending on the dose of porin proteins in the membrane (unpublished). Penicillins might be transported into the periplasm via two different pathways. Even so, it is obvious from this study that the permeation through the hydrophobic region plays an important role in ampicillin permeation in living cells. It will also be interesting to know the molecular basis of such a difference in outer membrane permeability between penicillins and cephalosporins.

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